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Alterations in Fatty Acid Composition of Murine Keratinocytes with In Vitro Cultivation

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The availability of methods for the in vitro cultivation of keratinocytes has spawned numerous studies utilizing these systems to analyze epidermal biochemical pathways, e.g., eicosanoid production. To determine whether these culture systems are indeed valid models for studies of eicosanoid products, we analyzed the fatty acid (FA) composition, especially of eicosanoid precursors linoleic acid (LA) and arachidonic acid (AA), of cultured and noncultured mouse keratinocytes. Neonatal mouse epidermal keratinocytes were cultivated in Dulbecco's modification of Eagle's medium + 10% fetal calf serum (FCS). Lipids of the cultivated cells, as well as noncultivated keratinocytes and whole epidermis were extracted in $CHCl_3$:MeOH (2:1) and lipid classes separated by thin-layer chromatography. The FA composition of the total lipid extract as well as of the phospholipid class was determined by gas-liquid chromatography of FA methyl esters. There was a gradual decrease in the LA levels in the cultured cells; by day 5 of culture the cells demonstrated a 4-fold ($p < 0.001$) decrease in LA as compared to either noncultured cells or whole epidermis. Levels of AA, on the other hand, remained unchanged during culture. Analysis of the FCS used in the culture medium revealed that the level of LA was 4-fold lower than that of normal mouse serum. Since LA is an essential FA which is not synthesized by the cell, the decreased LA in cultured cells probably results from the paucity of this FA in the FCS-containing culture medium. These studies indicate that keratinocytes cultivated in FCS-containing medium demonstrate profound alterations in levels of LA. Hence, in vitro keratinocyte studies dependent on cellular polyunsaturated FA substrates should be interpreted with caution. The relationship of altered cellular levels of LA on keratinocyte differentiation remains to be determined.

A number of recent studies have examined the role of eicosanoids, both the prostaglandins and the leukotrienes, in normal epidermal biology and in the pathogenesis of skin diseases such as psoriasis [1–7]. Some studies have utilized whole skin preparations [1,2,4], while others, in an effort to eliminate the contribution of dermal and blood cellular components, have utilized keratinocyte cultures as their models [3,5–7]. The murine [8,9] and human [10,11] keratinocyte culture systems, now in wide use, are generally accepted to be valid models for the study of epidermal biology. To consider the in vitro culture system a valid model for these eicosanoid studies, it is important to demonstrate that the fatty acid precursors, linoleic and arachidonic acids, are present within cultured keratinocytes in relatively the same amounts as in intact epidermis. The current study was therefore undertaken to determine the fatty acid composition, and in particular the percentage of linoleic and arachidonic acids, of cultured and noncultured murine keratinocytes. We have found that the amounts of linoleic acid change considerably during routine cell culture, probably reflecting the fatty acid content of the culture medium. Hence, studies investigating eicosanoid metabolism that utilize cultured cells must be interpreted with caution.

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Abbreviations:
DMEM: Dulbecco's modification of Eagle's medium
FAME: fatty acid methyl ester
FCS: fetal calf serum
GLC: gas-liquid chromatography
TLC: thin-layer chromatography

MATERIALS AND METHODS

Cell Preparation

Primary mouse keratinocyte cultures were established using the method of Yuspa and Harris [9]. Skin from neonatal BALB/c mice was floated in 0.25% trypsin in Hanks' balanced salt solution overnight at 4°C. The epidermal sheets were separated, disaggregated into a single cell suspension, filtered through mesh to remove stratum corneum, and plated on plastic culture dishes (Corning) at a density of 2×10^5 cells/cm^2 in Dulbecco's modification of Eagle's medium (DMEM) + 10% fetal calf serum (FCS). For some experiments, either the trypsin-separated epidermal sheet or the mesh-filtered epidermal cell suspension was directly analyzed for lipids without prior cultivation.

Lipid Extraction and Purification

Cultures were rinsed twice with phosphate-buffered saline, 1 ml of ice-cold MeOH was added to each dish, after which the cells were scraped into the MeOH with a rubber policeman and pooled. Chloroform was added to the pooled MeOH cell suspension to give a final ratio of $CHCl_3$:MeOH of 2:1. The cell suspension was homogenized with a ground glass homogenizer, and the method of Folch et al [12] was used to extract lipids. The lipid extracts were dried under a vacuum and stored in $CHCl_3$:MeOH (2:1) under N_2 at −20°C until analysis. Lipid extracts of fetal calf and mouse sera as well as mouse lab chow were similarly prepared and stored.

Lipid extracts were applied to activated Silica Gel G thin-layer chromatography (TLC) plates and chromatographed in one direction using $CHCl_3$:MeOH:acetic acid:H_2O (90:8:1:0.8, v/v). Bands of the different lipid classes were visualized by exposure of the plates to iodine vapors and identified by comparison of *Rf*'s to those of authentic standards. Neutral lipids were completely separated from all phospholipids by this procedure. To separate the free fatty acid fraction, total lipid extracts were chromatographed on Silica Gel G TLC plates using a benzene:ether:ethylacetate:acetic acid (160:20:20:0.4, v/v) development system. The free fatty acid fraction was scraped from the plate, eluted, and further separated from cochromatographing prostaglandins by TLC using an ethyl acetate:formic acid (208:2, v/v) solvent system.

Gas-Liquid Chromatographic (GLC) Analysis

Fatty acids from total lipid extracts or from phospholipid fractions were transmethylated using methanolic HCl as previously described [13], and the resultant fatty acid methyl esters (FAMEs) were extracted in petroleum ether and evaporated to dryness. Free fatty acid fractions were directly methylated using diazomethane-ether solution. The resultant FAMEs were analyzed on a Hewlett-Packard 5730A gas chromatograph equipped with a flame ionization detector system, using a fused silica capillary column (DB-225, J & W Scientific, Rancho Cordova, California). The column temperature was maintained at

200°C and the carrier gas flow rate was 1.76 ml/min. The FAMEs were identified by comparison of retention times to those of known standards (Nu-Check Prep, Elysian, Minnesota) and relative percentages were calculated by integration of the area under each peak using the Hewlett-Packard 3380A Integrator.

Materials

BALB/c mice and mouse chow "White Diet" were obtained from Simonsen Laboratories, Inc., Gilroy, California. Powdered DMEM and FCS were obtained from Flow Laboratories, Inglewood, California, trypsin 1:250 was obtained from Difco Laboratories, Detroit, Michigan. TLC and GLC solvents were all of analytical grade (Mallinckrodt). Pooled mouse serum was collected from 1-day-postpartum BALB/c mothers of the neonates used for epidermal culture.

RESULTS

Chromatographic analysis of the fatty acids present in the total lipid extracts of the noncultured keratinocytes and of those cultured for 5 days using standard FCS-utilizing methodologies [9] reveals marked changes in levels of linoleic acid (18:2n6) in these two cell populations (Fig 1). A 4- to 5-fold decrease of this fatty acid is observed in the cultured cells; the levels of arachidonic acid (20:4n6), on the other hand, appear equal in both groups of cells. Since cellular polyunsaturated fatty acids are preferentially esterified to membrane phospholipids, this lipid class was further analyzed. Table I shows the phospholipid fatty acid profiles from whole epidermis, noncultured cells, and cells cultivated for 5 days. The suspension of noncultured epidermal cells differs from the whole epidermis in that most of the stratum corneum has been removed by mesh-filtration. This may account for the observed decrease in longer-length fatty acids (22:0 and 24:0) in the noncultured cells as compared to the whole epidermis with cultivation. Levels of these fatty acids continue to decrease, probably as a result of the lack of nonadherent stratum corneum and granulosum cells, wherein most of the longer-chain fatty acids are esterified to the sphingolipids and neutral lipids [14]. A 2-fold increase in the level of 16:1 is also noted in the cultured cells. The most marked change observed, however, is again in the linoleic acid (18:2n6) fraction. A 4-fold decrease of this fatty acid is observed in the phospholipids of the cultured cells as compared to either the noncultured cells or the intact epidermis. Linolenic acid (18:3n3), another essential fatty acid, is also reduced in the cultured cells, though less significantly so ($p < 0.01$). Remarkably, the percentage of the third essential fatty acid, arachidonic acid (20:4n6), remained unchanged in all the epidermal preparations, accounting for approximately 4% of total fatty acids.

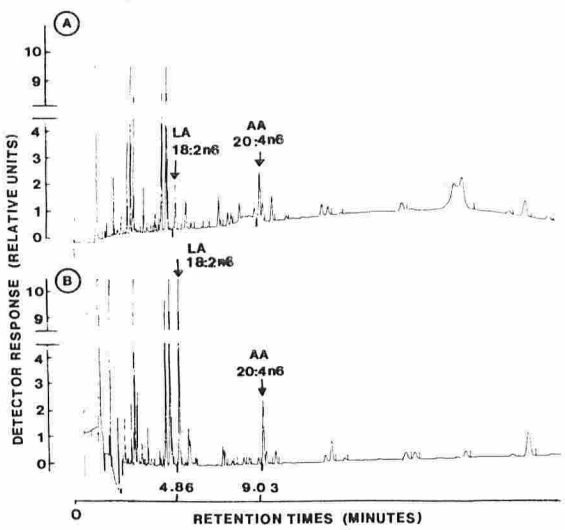

FIG 1. GLC of FAMEs present in total lipid extracts of keratinocytes. FAMEs were prepared as outlined in *Materials and Methods* and two representative chromatograms are shown. *A*, Keratinocytes cultured for 5 days in FCS-containing medium. *B*, Noncultured keratinocytes. *LA* = linoleic acid, *AA* = arachidonic acid. Detector response is given in arbitrary units; note break in scale.

TABLE I. *Fatty acid composition of mouse keratinocyte phospholipids expressed as weight % of total fatty acid methyl esters*

Fatty acid	Phospholipids		
	WE (n = 2)	NC (n = 4)	5DC (n = 4)
16:0	16.81 ± 1.38	20.24 ± 1.72	20.29 ± 0.59
16:1	3.10 ± 0.74	2.09 ± 0.21	5.44 ± 1.31
18:0	9.38 ± 0.56	12.33 ± 1.87	12.44 ± 1.53
18:1n9	17.33 ± 2.91	14.50 ± 2.71	17.30 ± 3.93
18:2n6	14.91 ± 1.43	15.78 ± 3.18	3.87 ± 0.69
18:3n3	0.90 ± 0.51	5.19 ± 1.01	2.75 ± 0.55
20:0	1.28 ± 0.14	1.80 ± 0.27	<0.1
20:1n9	0.22 ± 0.17	<0.1	1.03 ± 0.10
20:2	0.30 ± 0.78	<0.1	<0.1
20:3n9	<0.1	<0.1	<0.1
20:3n6	0.44 ± 0.48	<0.14	0.86 ± 0.18
20:3n3	0.18 ± 0.11	0.1	1.06 ± 0.16
20:4n6	3.93 ± 0.16	3.56 ± 0.98	3.50 ± 0.54
22:0	4.82 ± 0.33	3.74 ± 0.63	1.07 ± 0.06
22:1n9	<0.1	<0.1	<0.1
22:5n3	<0.1	<0.1	<0.1
22:6n3	<0.1	<0.1	<0.1
24:0	13.18 ± 0.05	7.17 ± 1.65	4.13 ± 0.13
24:1	<0.1	<0.1	<0.1

The fatty acid composition of the phospholipid class isolated from lipid extracts as described in *Materials and Methods* is given in percentage ± SD of total fatty acids in each group. WE = whole epidermis; NC = noncultured epidermal cell suspension prior to plating; 5DC = 5-day-old cultures, maintained as described in *Materials and Methods*. Only the major fatty acids are reported, therefore total does not equal 100%.

TABLE II. *Fatty acid composition of fetal calf and mouse serum expressed as weight % of total fatty acid methyl esters*

Fatty acid	Total lipids		Free fatty acids	
	FCS (n = 5)	MS (n = 4)	FCS (n = 4)	MS (n = 4)
16:0	21.59 ± 1.21	17.73 ± 2.34	31.64 ± 2.44	26.09 ± 2.74
16:1	4.05 ± 0.26	1.70 ± 0.12	<0.1	2.16 ± 0.77
18:0	11.72 ± 0.47	8.70 ± 1.03	19.69 ± 2.39	6.92 ± 4.21
18:1n9	18.96 ± 0.97	18.21 ± 2.58	4.91 ± 0.36	17.68 ± 0.7
18:2n6	4.61 ± 0.26	26.66 ± 2.07	7.60 ± 2.99	24.61 ± 0.66
18:3n3	0.31 ± 0.02	0.65 ± 0.17	<0.1	0.92 ± 0.07
20:0	0.64 ± 0.22	0.29 ± 0.05	<0.1	<0.1
20:1n9	0.29 ± 0.02	0.65 ± 0.05	<0.1	0.80 ± 0.08
20:2	<0.1	0.29 ± 0.03	<0.1	0.12 ± 0.04
20:3n9	0.50 ± 0.03	0.14 ± 0.04	<0.1	<0.1
20:3n6	1.77 ± 0.19	0.80 ± 0.12	<0.1	0.33 ± 0.27
20:3n3	<0.1	0.12 ± 0.05	6.06 ± 2.16	0.87 ± 0.88
20:4n6	7.19 ± 1.34	13.44 ± 4.06	<0.1	<0.1
22:0	1.67 ± 0.17	0.19 ± 0.05	<0.1	<0.1
22:1n9	<0.1	<0.1	<0.1	<0.1
22:5n3	2.67 ± 0.76	<0.1	<0.1	<0.1
22:6n3	3.85 ± 1.07	3.60 ± 1.64	<0.1	<0.1
24:0	1.14 ± 0.11	<0.1	<0.1	<0.1
24:1	2.36 ± 0.26	0.27 ± 0.19	<0.1	<0.1

The fatty acid composition of total extracted lipids or of the free fatty acid fraction is given in percentage ± SD of total fatty acids in each group. FCS = fetal calf serum; MS = mouse serum. Only the major fatty acids are reported, therefore total does not equal 100%.

Cultured cells incorporate fatty acids which are present within serum-containing culture medium [15]. Although the incorporated fatty acids preferentially derive from serum-free fatty acids [16], fatty acids may also be incorporated from the more abundant serum glycerides and phospholipids [17]. Therefore, the fatty acid compositions of both the total lipid and free fatty acid fractions of the FCS used in culture medium and mouse serum were determined (Table II). The level of 16:1 is increased in FCS as compared to mouse serum, which probably contributes to observed increase of this fatty acid in cultured keratinocytes. Linoleic acid accounts for a rather small per-

centage of both total and free fatty acids (4.6% and 2.4%, respectively) in FCS. In contrast, mouse serum, the source of this essential fatty acid in vivo, is quite rich in linoleic acid (~25% of total and free fatty acids). The level of arachidonic acid is likewise significantly higher in mouse serum as compared to FCS (13% vs 7%). Fig 2, constituted from Fig 1 and Table II, compares the relative percentages of 18:2n6 and 20:4n6 present in the epidermal and serum lipid extracts in histogram form. The significant ($p < 0.001$) decreases in levels of linoleic acid in the cultured cells and in FCS, as compared to intact epidermis and mouse serum, respectively, are easily visualized in this figure.

Serum essential fatty acids cannot be metabolically synthesized but are derived from dietary sources. Since our BALB/c breeding colony was fed a rodent chow especially formulated for breeders, ("White Diet"), we examined the fatty acid composition of this diet and compared it to the standard Purina rodent chow (Table III). Linoleic acid is a major fatty acid in both of these diets (27% of White Diet and 51% of Purina diet total fatty acids), hence the observed high concentration of this fatty acid in mouse serum probably reflects the dietary contribution.

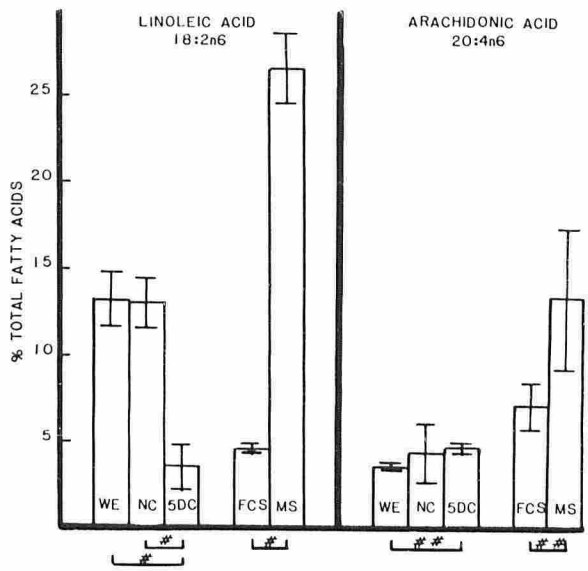

FIG 2. Percentages of linoleic and arachidonic acids present in total lipid extract of epidermal and serum preparations. The fatty acid compositions are taken from Fig 1 and Table II. *WE* = whole epidermis, *NC* = noncultured epidermal cells, *5DC* = 5-day-old cultures, *FCS* = fetal calf serum, *MS* = mouse serum, # = $p < 0.001$, ## = $p < 0.01$ (Student's two-tailed *t*-test).

TABLE III. *Fatty acid composition of mouse chow diet expressed as weight % of total fatty acid methyl esters*

Fatty acid	Purina[a]	White Diet[b]
14:0	2.30	0.15
16:0	20.53	13.13
16:1	3.33	0.27
18:0	9.27	2.73
18:1n9	30.54	28.21
18:2n6	27.25	51.10
18:3n3	3.07	2.77
20:0	0.26	0.44
20:1n9	NR	0.32
22:0	NR	0.26
24:0	NR	0.31

Only the major fatty acids are included, therefore, total does not equal 100%. NR = not reported.

[a] Purina Standard Rodent Chow #5001. Values represent percent of total fatty acids and are supplied by the Ralston Purina Co., St. Louis, Missouri (personal communication, D.C. Shelton, Director, Special Chows Research).

[b] "White Diet" rodent chow is a product of Simonsen Laboratories, Inc. Values represent percent of total fatty acids in the total lipid extract, determined as outlined in *Materials and Methods*.

DISCUSSION

With recent advances in keratinocyte cell culture these in vitro systems are being used more frequently to study specific biochemical aspects of epidermal biology. They offer the advantages of a pure keratinocyte population which demonstrates many features of normal, in vivo differentiation, and therefore they are generally accepted to be valid models of the in vivo epidermis. The current study demonstrates one major biochemical alteration in routinely cultivated (i.e., in FCS-supplemented medium) murine keratinocytes: a profound decrease in the content of the essential fatty acid, linoleic acid (18:2n6), as compared to normal mouse epidermis. Since linoleic acid and its metabolite arachidonic acid are precursors for the generation of the biologically potent prostaglandin and leukotriene products, the numerous studies measuring the generation and effect of these products utilizing cultured keratinocytes [3,5–7] must be interpreted with caution. The currently demonstrated decrease in substrate (18:2n6) availability in cultured cells as compared to the in vivo situation may significantly hamper the generation of eicosanoid products in the in vitro system.

The observed 4-fold decrease in linoleic acid content over a 5-day culture period (Table I) is the most dramatic change observed in the relative concentrations of keratinocyte essential fatty acids. There is a less remarkable, 2-fold decrease ($p < 0.01$) in the concentration of another essential fatty acid, linolenic acid (18:3n3). Arachidonic acid, on the other hand, remains a constant 4% of total fatty acids in both normal epidermis and cultured cells. The decreases in linoleic and linolenic acids observed during culture are gradual: on day 1 of culture the percentage of linoleic acid in keratinocyte phospholipids decreased from 15.8% in noncultured cells to 11.3%; by day 4 of culture it was 4.3% (data not shown). The stable level of keratinocyte arachidonate (4% of total fatty acids) during culture may indicate that this fatty acid is incorporated from the FCS in the culture medium rather than synthesized de novo. The current data do not indicate whether the keratinocytes have the metabolic capacity to transform the precursor linoleic acid into arachidonate. Additionally, the observed decrease in cellular linoleic acid with cultivation is apparently not unique to keratinocytes: Lagarde and coworkers have demonstrated similar alterations in fatty acid composition of human endothelial cells cultured in FCS-containing medium [18], as have Loomis and colleagues in cultured rat splenocytes [19].

Aside from alterations in the capacity of cultured keratinocytes to synthesize eicosanoids as a result of the decrease in the pool of precursor fatty acids, there are a number of other possible biologic pertubations that may result. Cell membrane fluidity is influenced by the amount of unsaturated fatty acids esterified to membrane phospholipids [20], and the observed 4-fold decrease in linoleate in cultured keratinocytes, may, likewise, alter keratinocyte membrane fluidity and perhaps function. Some cellular functions known to be altered with changes in membrane fatty acids include concanavalin A agglutinability [21], cellular adhesion [20], differentiation [22], and cell shape [23]. It is reasonable to suggest that the observed decrease in essential fatty acid composition of cultured cells may be, in part, responsible for the incomplete differentiation pattern observed in vitro. These questions require further study. The present study suggests however that the in vitro culture of murine keratinocytes in FCS-containing medium, though convenient, may lead to marked biochemical differences from the normal in vivo state.

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